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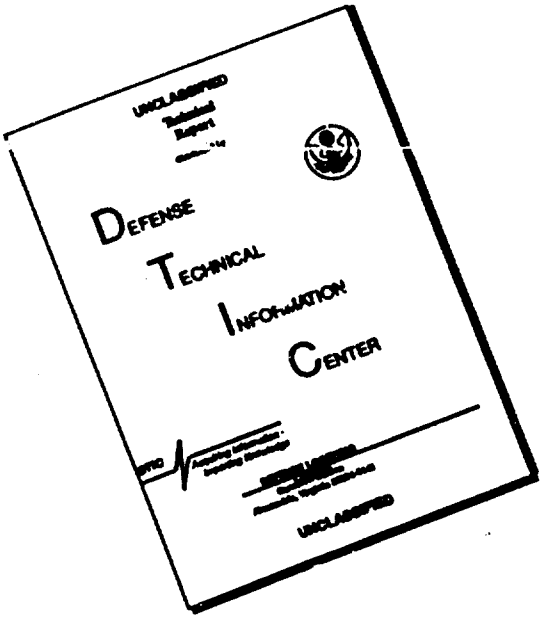

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THE INTERFERENCE PHENOMENON IN DENGUE VIRUS AND VEE VIRUS ON KB STABLE CELLS AND CHICK EMBRYO FIBROBLASTS

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Dr. A. L. Briceno Rossi

During the recent dengue epidemic in the eastern states of the republic, we obtained a virus diagnosis with the help of hemagglutination inhibition tests, using dengue "2" as the viral antigen; we compared the sera taken during the acute period and the convalescent sera in order to get an idea of the increase in antibodies between the two sera; this would give us an idea which of the two would have the better diagnostic value (Briceno Rossi, 1964).

Similarly, we inoculated the blood from patients in the 1-day lactant rat in order to isolate the virus (Albert B. Sabin, 1959); we did not obtain a satisfactory result. This is why it seemed to us that the dengue virus in this epidemic is not capable of making the lactant [suckling] rat sick; this is true in spite of the fact that we performed passages from one rat to the next.

In our endeavor to identify the presence of the dengue virus in this epidemic, we therefore conducted the study which we are reporting here, basing our research on two rather very important elements: one of them involved the growth and chronicity of the "2" virus in the stable "KB" cultivation cell, without producing pathogenic lesions in them [it] (Beasle and Associates, 1961); on the other hand we were quite familiar with this operation because we used this host cell system and the Polio I (Mahoney) virus which, in contrast, destroyed these cells in a short time.

Similarly, we remembered the work done by Buescher and Packman at Walter Reed Army Institute of Research (1962) on the virus diagnosis of rubeola by means of the interference phenomenon in the presence of the virus Echo II; we also recalled the studies by Sever of Bethesda, and Weller and Neva of the School of Public Health of Harvard University; we used the same quality of the rubeola virus to grow in the tissue medium [culture] without cytopathogeny and the destructive action of other viruses in the same cells in order thus to get an

idea of the presence of this virus with the help of the interference phenomenon.

As study material we used the following: the sera taken during the first 3 days of the infection period from clinical dengue cases which, since they contained virus particles, had to constitute the interference virus; as comparison virus [the virus that is interfered in] we used the Polio I virus (Mahoney).

Methods and Techniques Used

Tissue cultures of KB (Eagle) cells in tubes, controlled prior to processing, with a well-formed strain (these cells, which we used in the routine development of our cultures, originally came from "Microbiological Associates Bethesda," (Maryland).

For each serum studied, we used 12 tubes, subdivided as follows: 4 for cell control; 4 for the comparison virus control (Polio I); and 4 for the virus inhibition test (infectious serum but comparison virus).

In each test we placed 0.2 ml of clinical serum, per tube, as the interfering virus; this clinical serum was taken between the 1st-3rd days, starting from the beginning of the dengue infection; as comparison or "interfered" virus we took 100-200 TCD50 Polio I (Mahoney).

We then proceeded in the following manner: to the test tubes we add 0.2 ml of the problem serum (the infectious dengue serum) and we leave this in close contact for 2 hours, incubating at 37°C; the tubes are then washed with S. S. B. Earls; during the third washing we add 1 ml of sustaining medium and we place in the incubator at 37°C for a period of 48 hours. After this period of time has expired, we add, to the test tubes and to the polio control tubes, 100-200 TCD50 Polio I; we once again incubate at 37°C for another 48 hours in order to read off the results; on some occasions, we prolonged this for 3-4 days.

In order to get greater reliability, we performed the same tests with dengue 1 and 2 virus which we knew from those which had become acclimatized to the lactating rat; we had obtained these viruses from the C.D.C. Atlanta, Georgia Laboratories; we also used viruses from the "Trinidad Virus Lab," Trinidad, Pto. Espana; we used this virus in developing our dengue antigens for our routine serological work.

The first lot of sera studied came from students at the National Nurses' School, which had a dengue clinic, and who had been infected in the epidemic zone of Maturin, in the state of Monages. All of the sera were taken on the 2nd day day after the start of the infection. These sera were called 577, 578, 579, and 580-64.

As we can see in Table I, the results of the dengue 1 and dengue 2 virus cultivation (the previously mentioned known strains) revealed a limited inhibition of the Polio I [virus] with the dengue 1 virus as the interference in Polio [virus] was complete with the dengue 2 virus.

The results obtained with the sera from the clinical cases of dengue [fever], numbered 577 and 578-64, showed that the polio was inhibited and did not increase in the tubes which contained these sera; this indicated that this serum did contain the dengue virus which did interfere with Polio I; on the other hand, in the tubes containing only polio and in cases 579, 580-64, the cells were destroyed; this demonstrated to us the marked power of inhibition of sera 577 and 578-64 in the presence of the Polio I virus itself (see photos 578, 577, 579, and 580-64, with the respective controls).

This rather striking result indicated to us that we had found a simple method here in order to perform a virus diagnosis of dengue fever in clinical cases in a short time, let us say 4-5 days, without having to wait for the convalescent serum which would case a delay averaging 10 days in order to get the serum and thus to perform the previously mentioned I. H. tests. Next we had four new cases of dengue fever in the National Nurses' School; sera were taken during the first 3 days after the start of the infection process; one of them was a typical case (in other words, a textbook case, according to Dr. Hernandez, the epidemiologist who took this sample which is numbered 572-64; these were processed against others numbered 567, 568, 571-64; in this case, serum 572-64 clearly revealed interference.

TABLE I
RESULTS

October 1964

Interfering virus	Comparison virus	Host system, tissue culture	Results
Dengue 1, acclimatized to the rat	Polio I (Mahoney)	KB (Eagle)	+
Dengue 2, acclimatized to the rat	Polio I (Mahoney)	KB (Eagle)	+
Suspect serum, Dengue 442-64	Polio I (Mahoney)	KB	-
Suspect serum, Dengue 514-64	Polio I (Mahoney)	KB	-

+ Interference
- No interference

Continuing our study of the problem of diagnosing dengue from the viral viewpoint, we subjected new sera, numbered 645-64, 648-64, 649-64, and 692-64, to the same test; these sera come from the zone of Rios Carine, near Carupano (the infected zone), with dengue and EEV. As in all of the earlier cases, we inoculated lactant rats in our operating routine with the serum taken during the period of infection; this is why we were surprised when the sera in cases 645-64 and 649-64 caused manifestations of encephalitis in lactant rats within 24 hours;

because of the short incubation period, this should have come with another virus. Several days later, while conducting the tests with the inhibition phenomenon with the KB host cell system, using the same confrontation [comparison] technique with Polio I (Mahoney), we obtained the same optimum results with the sera 645, 649-64 (see Table IV, below); in other words, we obtained perfect interference (see the photos and the controls).

TABLE IV
VENEZUELAN ENCEPHALIC VIRUS

6 November 1964 - 14 November 1964

Interfering virus	Comparison virus	Host system, tissue culture	Results
Suspected serum, encephalitis, SI-VEE No. 645-64	Polio I	KB	+
Serum, acute dengue, No. 648-64	Polio I	KB	-
Serum, encephalitis case, serum I with virus VEE No. 649-64	Polio I	KB	+
Serum, Dengue (acute) No. 692-64	Polio I	KB	-

+ Interference
- No interference

As we look at this table and the inoculation of the lactant [suckling] rat, simultaneously, we can distinguish the presence of the EEV virus as, in the suckling rat, the latter produces encephalitis within a rather short period of 24 hours, whereas in the case of dengue the situation is different (in the case where the suckling rat is infected, the incubation period is more than 6 days). In order to determine whether we were really dealing with the EEV virus, we performed the neutralization test on the rats, using sera 645 and 649-64; the result was positive with Venezuelan VEE.

In this case the interference virus was the Venezuelan VEE and the interfered or confronted [compared] virus was Polio I.

Now that we knew that the system of stable host cells in the KB (Eagle) tissue culture was successful, we immediately used the same techniques but employed a different host cell system (culture of fibroblasts of embryos of primary polio cells [sic; primary chick embryo cells]); on that occasion we performed the tests, for greater reliability, using the mixovirus A 2 (the Venezuelan strain isolated by us in 1956) as well as the PR8, inactivated by means of heat, at 56°C for a period of 30 minutes; we also used the liquids from the cultures obtained with the KB tests, which correspond to the EEV virus, in cases 645, 649-64, where the results revealed active interference against Polio I (Mahoney).

For these tests we used the EEV virus (Maracay strain) as confrontation or comparison virus; it destroys the chick embryo fibroblast cells within 24-48 hours (Briceño Rossi, 1963).

In these last tests, we provided direct contact between the inactivated and liquid virus A2 and PR8 from the cultures (cases 645-64) and the chick embryo fibroblast cells for a period of 2 hours at 37°C; we washed the cells and we added 1 ml of the maintenance medium of these cells for 24 hours at 37°C; then we compared this with the Venezuelan encephalitis virus 100 MLD50 (Maracay strain) which we were quite familiar with; we used an equal dose for the EEV virus control tube; in each case we used the four tubes as normal control cells [sic].

We made the first reading 24 hours after adding the confrontation virus and then 48 hours later; the results after 48 hours can be seen in Table V and in the photos for this particular group of tests. Here we can see that both the Mixovirus Influenza A 2 (Venezuela strain) and the liquids in which we presumed have interference (645, 649-64) revealed the inhibition of the EEV virus as the result of these tests (clear interference); on the other hand, we can see that, when we used the inactivated PR8, interference with the Venezuelan encephalomyelitic virus was limited; in the tubes with the EEV virus only, the tissue revealed clear cytopathogenic lesions with destruction [of cells] (see Table V and photos).

On the basis of these inhibition studies, involving a short-time test and using stable KB cells for the dengue virus and the VEE (interfering virus) and Polio I (as comparison or interfered virus and in the primary fibroblast cells of the chick embryo, involving embryos 10 days old) for the case of the EEV virus and the Influenza A 2 virus as well as the PR8 inactivated virus, and on the basis of our observations of the compared or interfered VEE [sic] virus, it seems to us that this work can be catalogued under the phenomenon of viral interference; we are inclined to say this in accordance with the basic postulates of modern virology, concerning the estimate of viral virulence (Dulbeco, 1960) and the interpretations of inhibition in the case of viral superinfections (Henle, 1950; Isaacs, Lindeman, 1957, Isaacs, 1962; Burke and Isaacs, 1960); we would also say this on the basis of the recent studies on interference, using the arbor virus (R. Blaskovic, 1962). We also think that we can use these findings in order to demonstrate rather quickly the presence of the dengue virus in the blood of clinical cases in this viroesis. Investigations on viral diseases have shown that superinfection by one and the same or by a different virus will diminish the severity of the infection and will inhibit its multiplication in the cell. The recovery phenomenon is similar to the immunological state from crossed protection agents; in present-day virology this is called the viral interference phenomenon (Isaacs, 1957). In a definition of this phenomenon, Schlesinger offers two points dealing with the interpretation of its action: one of them concerns "the inhibition of the multiplication of the virus in the host system which was infected simultaneously on another virus" and the other concerns "the suppression of damage, sickness, or death due to a virus due to a virus by means of the simultaneous infection of [by] another virus."

TABLE V
USING CHICK EMBRYO FIBROBLAST CELLS

Interference Virus	Host System	Comparison Virus	Control cells, Host system	Control VEE	Interference results
Liquid culture in KB with VEE and Polio I	Fibroblastic, chick embryos	V.E.E. (100-200 LD50)	Normal	Destroyed (100-200 LD50)	+
Liquid culture in KB with VEE and Polio I Case 645-64	Fibroblastic, chick embryos	V.E.E. (100-200 LD50).	Normal	Destroyed (100-200 LD50)	+
KPS inactivated at 56°C for 30 minutes	Fibroblastic, chick embryos	V.E.E. (100-200 LD50).	Normal	Destroyed (100-200 LD50)	+
Influenza A 2 (Venezuela)	Fibroblastic, chick embryos	V.E.E. (100-200 LD50	Normal	Destroyed (100-200 LD50)	+

+ Interference
- No interference

This protection phenomenon has been identified with the arbor virus (yellow fever in monkeys, Hoskins, 1935) in the clinic of viral diseases and in experimental observations on animals, in chick embryos, and recently in tissue cultures. This was also proved with rats in the case of the yellow fever virus (Findlay and Mac Callan, 1937) and with the encephalitis virus transmitted by sheep ticks from Central Europe (Blaskovic, 1962). This was furthermore proved with the influenza virus and the western horse encephalitis virus (Vilcek and Hirst); it was furthermore demonstrated by Lenne and Koprowki in a tissue culture, with virus transmitted by arthropods (in these studies the virulence is expressed in the form of cytopathogenic lesions and researchers were able to establish a viral inhibition through the absence of these lesions which had been interpretted by all as a viral interference phenomenon). This phenomenon is the result of an interaction [inter-reaction] between virus cells [between the virus and the cells] and because of this the cells provide a new protection factor which Isaacs and Lindeman had called interferon; this factor is different from the antibodies since it is not blocked by the immune sera, nor is there any direct action against the viral agent outside the cells; it is found in a larger or smaller concentration in the liquids which wash these cells. In the series of studies on virus interference it has been possible to prove that all of the animal cells are capable of producing this "interferon"; this was demonstrated in chick embryo cells, in the cells of ducks, rats, in rat tissue [cultures], in guinea pigs, rabbits, dogs, pigs, sheep, cows, monkeys, and man; as far as the virus is concerned, with which the formation of this interferon was accomplished, there are many such viruses, such as the polio virus, Mixovirus (influenza, para-influenza), the measles virus, the antivariolic vaccine, the herpes vaccine, the Roux sarcoma virus, the rat paliema [sic] virus

and the arbor virus. Isaacs (1962) said that "it seems reasonable to conclude that the production of interferon appears to be a general response by the cells of the vertebrates against infectious viruses."

Viruses transmitted by arthropods (arbor virus) hold a special position with respect to the virus interference phenomenon and easily lend themselves to the study of these phenomena; many of them produce very slight lesions which are not lethal in the tissue cultures which we use as a simplified system for the observation of the phenomenon; on other occasions the C.P.E. lesions appear delayed, compared to the other animal viruses, as in our case where we used the KB (Eagle) stable cells and the VEE [Venezuelan horse encephalitis], virus, which takes 3 days to bring out its lesions in these cultures. On the other hand, the arbor viruses appear to be much more sensitive to the action of the interferon and they would therefore appear to be quite adaptable to the interference phenomenon.

"The formation of the interferon induced by the interference virus represents the only known mechanism through which interference can be established by viruses transmitted by arthropids" (Blaskovic, 1962). The interpretation of the phenomenon of earlier inhibition in our cases of dengue, EEV, influenza A 2 virus and PR8 virus and in the liquids in cases 645, 649-64 (where we presumed the presence of an interferon) can be explained only as viral interference phenomenon; the test was performed with stable KB cells and primary chick embryo cells.

At this point we want to express our appreciation to Miss Helena Segura Diaz who helped us in this project and to the photographer of the National Hygiene Institute, Heriberto Barrueta, who did the microphotography work.

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FIGURE CAPTIONS

[Page 37 of original] Legend: a — KB — infectious dengue serum — Polio I, case I - 578-64, interference; b — KB — infectious dengue serum - Polio I, case II - 577-64, interference; c — KB - infectious dengue serum — Polio I, case III, 579-64, no interference; d — KB + infectious dengue serum - Polio I, case IV, 580-64, no interference; e — KB control cell; f — KB - Polio I.

[Page 39 of original] Legend: a — KB + infectious dengue serum + Polio I, case 572-64, interference; b — KB - horse encephalitis serum, Venezuelan (VEE) - Polio I (Mahoney) case 645, interference; c — KB - serum I Venezuelan horse encephalitis (VEE) - Polio I (Mahoney), case 649, interference; d — KB control cell; e — KB - Polio I.

[Page 43 of original] Legend: a — virus interferes, mixed virus strain, A 2 (Venezuelan 1956) in chick embryo fibroblast cells, 24 hours, at [illegible] °C, [illegible word] virus compared VEE (Maracay strain), interference; b — 9R8 [sic], inactive, 36°C [illegible] for 30 [minutes], added for 2 hours in [to] chick embryo fibroblasts and compared after 24 hours with VEE (Maracay), limited interference; c — interferon, case 645 (VEE) in KB, plus VEE (Maracay) in chick embryo fibroblast cells, interference; d — interferon, case 649, VEE in KB compared with virus VEE (Maracay) in chick embryos fibroblasts, interference; e — control cell, chick embryo fibroblasts, November 1964; f — VEE control virus (Maracay strain) in chick embryo fibroblasts.